

Alteration of Glucose Transport and Diauxic Growth in 5-Thio-D-Glucose-Resistant Mutants of *Azotobacter vinelandii*†

DENISE MCKENNEY AND THOYD MELTON*

Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695

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Spontaneous mutants of *Azotobacter vinelandii* defective for glucose utilization were selected as resistant to 5-thio-D-glucose. Mutant strains AM2, AM38, and AM39 exhibited longer generation times than the wild type when grown on glucose. Mutant strain AM2 also exhibited an altered K_m and V_{max} for glucose uptake. During acetate-glucose diauxie, glucose utilization in the 5-thio-D-glucose-resistant mutants was subject to severe inhibition by acetate. These mutants did not exhibit the normal glucose phase of diauxie. Transport studies during diauxie indicated that glucose uptake was not induced in mutant strain AM2. However, increasing the glucose concentration from 25 to 200 mM relieved the severe acetate inhibition, and under these conditions the mutant strain AM2 exhibited normal diauxie. Revertants of mutant strain AM2 exhibited normal glucose and diauxie growth. The results are discussed in terms of a model for acetate regulation of glucose utilization in *A. vinelandii*.

We have been interested in obtaining *Azotobacter vinelandii* mutants that are defective for acetate or glucose utilization or both to better understand the regulation of acetate-glucose diauxie described for this organism (4). *A. vinelandii* organisms can utilize glucose efficiently for growth. The biochemistry of glucose transport in membrane vesicles of this bacterium has been described (1-3). The active transport of D-glucose by membrane vesicles of *A. vinelandii* O is coupled to the oxidation of L-malate via a flavin-linked L-malate dehydrogenase (1, 3). The glucose carrier expressed in vesicles from glucose-grown cells is highly stereospecific and is induced by growth of cells on D-glucose (1, 3). Membrane vesicles prepared from cells grown in the presence of acetate accumulate glucose at a more reduced rate than do vesicles from glucose-grown cells (3). These studies have provided some detail about the biochemistry of the glucose transport system in membrane vesicles of *A. vinelandii*; however, no information to date exists on the molecular biology of the in vivo components of this system. Success in this endeavor has been greatly hampered by the lack of proper genetic tools to explore the system. We have recently developed specific selection techniques to isolate mutants defective for glucose transport in *A. vinelandii*. Mutations in this system should provide a means to identify the gene(s) and gene product(s) required for its expression. In this study we describe the isolation and characterization of *A. vinelandii* mutants resistant to 5-thio-D-glucose (TG).

In mammalian cells, TG affects transport of glucose, galactose, and neutral amino acids such as glycine (6, 17) and inhibits hexokinase (13). In enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium*, TG affects the phosphoenolpyruvate-dependent phosphotransferase transport system (7). *E. coli* mutants resistant to TG can still grow on glucose but only half as well. Glucose is no longer used in preference to other sugars (11), but rather is used simultaneously with other sugars, and adenylate cyclase activity is

lower than in wild-type *E. coli* (10). The TG-resistant *E. coli* strains are strongly impaired in their ability to adapt to growth on C₄-dicarboxylic acids as sole carbon sources. This impairment is overcome by adding cyclic AMP to the media (5). Two genes are involved in TG resistance in *E. coli*. One gene, designated *tgs*, maps between *cysA* and *ptsI*, whereas another gene specifying loss of inducer exclusion by glucose maps near *ptsI*.

MATERIALS AND METHODS

Chemicals. TG was obtained from Sigma Chemical Co. The uniformly ¹⁴C-labeled substrates used in transport studies were [1,2-¹⁴C]acetic acid (56.5 mCi/mmol), from New England Nuclear Corp., and [D-¹⁴C]glucose (263 mCi/mmol) from Amersham Corp.

Bacterial strains and culture conditions. *A. vinelandii* OP was used as the wild-type strain for these studies. Growth conditions were the same as those previously described (12). Cells were routinely grown at 28°C in Burk nitrogen-free media (BM) (15) and usually washed in Burk buffer (15).

Isolation of spontaneous mutants and revertants. Mutants defective for glucose utilization were selected as spontaneous TG-resistant colonies growing on a BM-2% fructose plate containing 10 mM TG. Wild-type *A. vinelandii* cells were spread on BM-fructose plates with 10 mM TG and allowed to incubate for 4 to 7 days at 28°C. TG-resistant mutants AM2, AM38, and AM39 were isolated and further characterized. Revertants strains AM51 and AM52 were selected by growing the TG-resistant mutant strain AM2 overnight in liquid BM-2% glucose medium before plating on BM-2% glucose plates.

Transport studies. Transport studies were performed as described previously by McKenney and Melton (12).

RESULTS

Effects of TG on growth of wild-type *A. vinelandii*. TG is an analog of glucose. The presence of 10 mM TG completely inhibited growth of wild-type *A. vinelandii* on fructose (Fig. 1) as well as on sorbitol. Growth of *A. vinelandii* on glucose was not inhibited by TG. Also, when 25 mM glucose was present in media containing 100 mM fructose, growth was

* Corresponding author.

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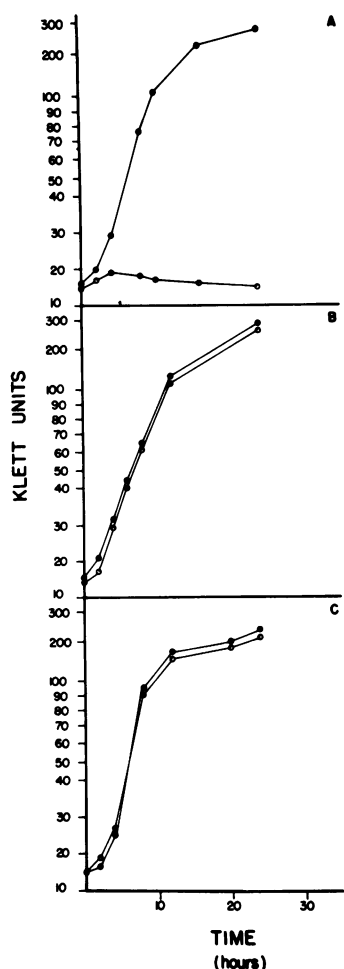


FIG. 1. Growth of wild-type *A. vinelandii* with (○) and without (●) 10 mM TG. Cells were pregrown in BM-2% sucrose at 28°C with aeration. Cells were washed with Burk buffer and used to inoculate flasks containing medium and (A) 100 mM fructose, (B) 100 mM glucose, or (C) 25 mM glucose plus 100 mM fructose.

not inhibited by TG (Fig. 1). However, as the concentration of glucose in fructose media was decreased, the level of protection from inhibition by TG also decreased. No protection was conferred by the glucose analogs α -methyl-glucoside or 2-deoxy-glucose. Growth of wild-type *A. vinelandii* on maltose, benzoate, acetate, or galactose was not inhibited by TG. However, growth on sucrose was partially inhibited by TG.

Isolation of TG-resistant mutants of *A. vinelandii*. Growth of wild-type *A. vinelandii* on fructose was completely inhibited by TG at a concentration of 10 mM. At 2 mM TG slight growth (less than one doubling) occurred over a 24-h period in liquid medium. TG-resistant mutants were isolated as spontaneously resistant colonies on BM-fructose plates containing 10 mM TG. When wild-type *A. vinelandii* cells were spread on BM-fructose plates containing 10 mM TG, large colonies appeared upon a background growth of many small colonies. The background growth was greatly reduced when agarose was used to prepare the plates instead of agar. Three TG-resistant mutants, designated AM2, AM38, and AM39, were selected for further characterization.

The growth characteristics of the TG-resistant mutants was determined on a variety of carbon sources. Growth of

the TG-resistant mutants was impaired on glucose. Generation times on glucose media were 10.5 h for strains AM2 and AM38 and 11.5 h for strain AM39 compared with 3.5 h for the wild type. Growth on maltose was also impaired in the TG-resistant mutants. These strains exhibited a generation time of 7 h on maltose compared with the 5 h for the wild type. Growth of the TG-resistant strains was normal on fructose, galactose, sucrose, sorbitol, mannitol, and xylitol. Mutant strain AM2 exhibited slow growth on glucose liquid media; however, when the cells were washed and suspended in fresh glucose medium no growth was detected.

Uptake of glucose by mutant strain AM2. Since the TG-resistant mutant AM2 failed to grow well on glucose as the sole carbon source, we examined the transport of radioactive glucose in this mutant. Uptake of glucose was significantly decreased in mutant strain AM2. The mutant strain AM2 had a K_m of 0.47 mM for glucose uptake compared with a K_m of 0.15 mM for glucose uptake in the wild type. The V_{max} for glucose uptake in mutant strain AM2 was 0.83 nmol/min per mg of cell dry weight, whereas for the wild type the V_{max} was 2.22 nmol/min per mg (Fig. 2). Poor growth of mutant strain AM2 on glucose can be related to altered transport kinetics for glucose. Transport of fructose and acetate was essentially the same for both mutant AM2 and wild-type *A. vinelandii*.

Uptake of acetate and glucose in mutant strain AM2 during diauxie. *A. vinelandii* exhibits diauxic growth in media containing both acetate and glucose. Under these conditions

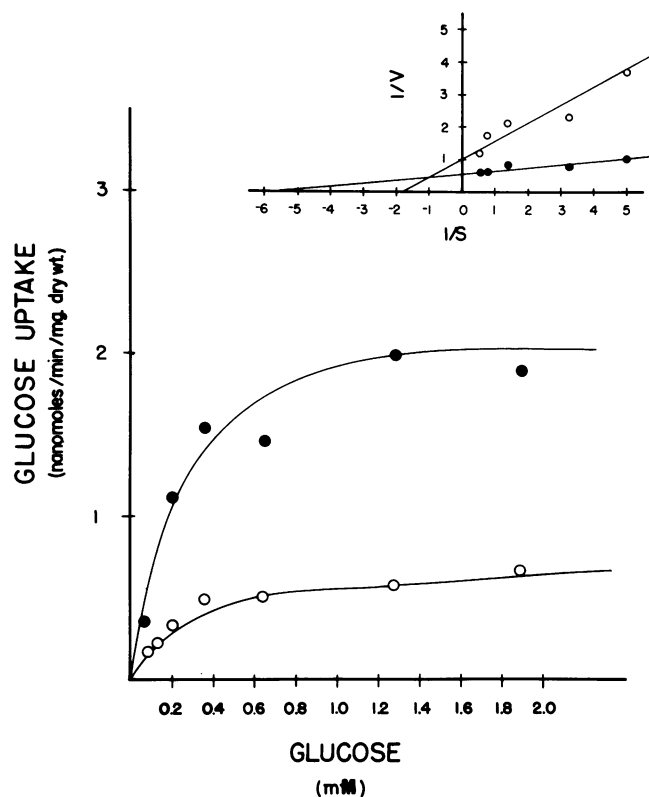


FIG. 2. Kinetics of [14 C]glucose uptake in wild-type *A. vinelandii* and AM2. Cells were pregrown in BM-2% sucrose before inoculation in BM-2% glucose and were prepared as described in Materials and Methods. Uptake was measured at 28°C in the wild type (●) and AM2 (○). The inset is a double-reciprocal plot of the same kinetic data.

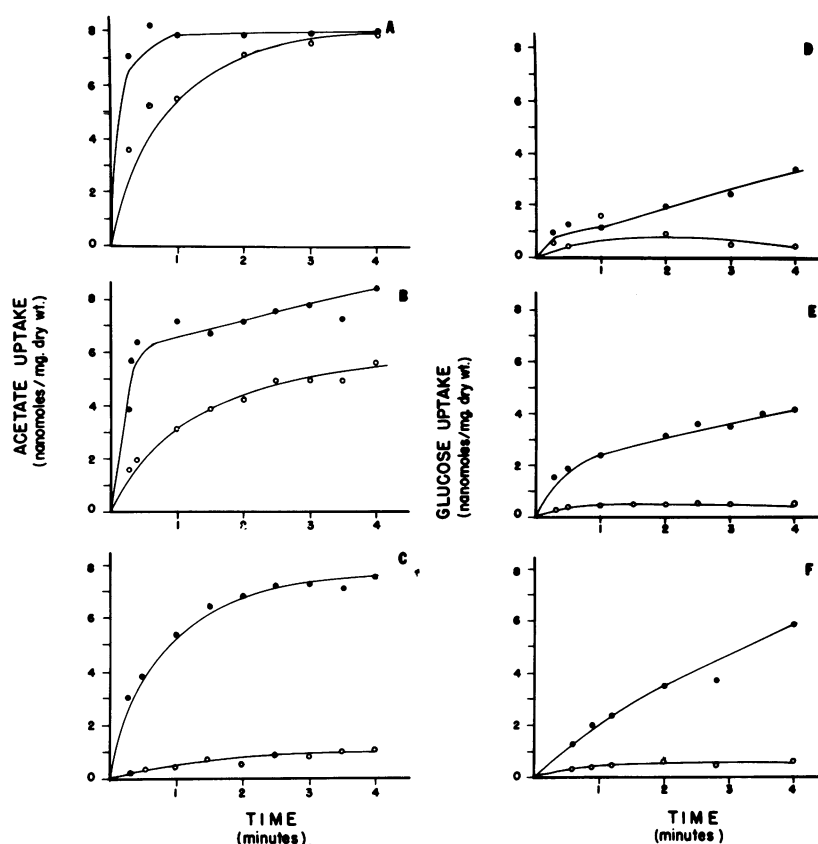


FIG. 3. Time course of [^{14}C]acetate (A, B, C) and [^{14}C]glucose (D, E, F) uptake after 6 h (A, D), 12 h (B, E), and 18 h (C, F) during acetate-glucose diauxie. Cells were pregrown in BM-2% sucrose with 30 mM ammonium acetate overnight, harvested, washed twice in Burk buffer, and suspended on 45 mM acetate-25 mM glucose. The uptake of the sugars (63 μM) was measured at 28°C in the *A. vinelandii* wild type (●) and mutant AM2 (○).

acetate is the preferred carbon source (4). Even though the TG-resistant mutants grew slowly on glucose alone, during diauxie the metabolism of 45 mM acetate inhibited growth on glucose. The same effect was observed in acetate-maltose media. Acetate was also found to inhibit the growth of TG-resistant strains when added to cultures growing on glucose alone. The TG-resistant mutants were permanently inhibited for glucose utilization in the presence of acetate, whereas the wild type was only transiently inhibited. We examined acetate and glucose uptake during diauxie for the wild type and mutant strain AM2 (Fig. 3). Initially, transport of acetate was similar in both the wild type and the TG-resistant mutant AM2 during the first 6 h of diauxie (Fig. 3A). The ability of strain AM2 to transport acetate decreased during the next 12 h of diauxie (Fig. 3B and C). Unlike glucose transport in the wild type, glucose transport was never induced in the TG-resistant mutant AM2 under diauxie conditions (Fig. 3D through F). However, increasing the concentration of glucose in the acetate-glucose diauxie media circumvented acetate inhibition of the glucose system. The usual concentration of glucose used in diauxie media was 25 mM. Increasing the glucose concentrations to 50 or 100 mM did not relieve acetate inhibition or induce the glucose system. However, when the concentrations of glucose were increased to 200 mM, mutant AM2 exhibited a characteristic transient diauxie lag which was followed by the characteristic glucose phase of growth. Interestingly, the wild-type *A. vinelandii* also showed a response to the elevated levels of glucose during diauxie. The wild type

under these conditions did not exhibit a characteristic diauxie lag. The implications of this observation are discussed below.

Revertants of TG-resistant mutant strain AM2. When mutant strain AM2 was pregrown on glucose minimal medium (for which it has a long generation time), washed, and transferred to minimal glucose plates, small and large colony types appeared. We reasoned that the large colony type was a revertant having growth characteristics of the wild-type strain. Two strains, AM51 and AM52, were selected and further characterized. Both of these strains exhibited normal growth on glucose (Fig. 4A). The generation time for these revertants on glucose was the same as for the wild type. Not only were these revertants able to grow on glucose normally, but they also exhibited diauxie growth (Fig. 4B). This suggests that the process by which glucose metabolism is usually induced during diauxie had been regained by these revertants.

DISCUSSION

TG is an analog of D-glucose which has a sulfur atom in place of an oxygen atom in the pyranose ring. This analog affects the transport of glucose in eucaryotic and procaryotic cells and affects the transport of galactose and neutral amino acids such as glycine in mammalian cells (6, 13, 17). *Escherichia coli* mutants have also been selected as resistant to TG and were defective for glucose growth and no longer used glucose in preference to other sugars (5, 7-9, 11). Adenylate

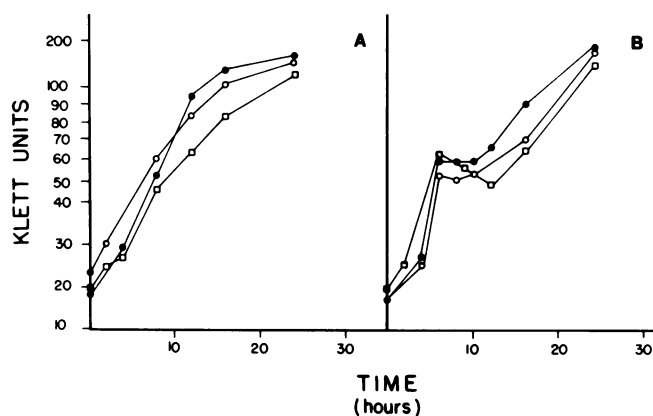


FIG. 4. Growth of wild-type *A. vinelandii* and revertants on glucose and under diauxic conditions. Cells were pregrown in BM-sucrose before inoculation into glucose medium (A) or were pregrown in BM-2% sucrose with 30 mM ammonium acetate before inoculation into 45 mM ammonium acetate-25 mM glucose medium (B). Symbols: (●) wild-type *A. vinelandii*, (○) AM51, (■) AM52.

cyclase activity was also lower in the *E. coli* TG-resistant mutants (10).

In this paper we describe the isolation and characterization of TG-resistant mutants of *A. vinelandii* that are defective for glucose transport. Wild-type *A. vinelandii* was sensitive to TG when grown on fructose; however, when glucose was present in TG-fructose media, cells were no longer sensitive to TG. Except for sucrose no other growth substrate tested induced TG sensitivity in *A. vinelandii*. In *A. vinelandii* glucose is actively transported (1, 2), and the glucose carrier is highly stereospecific (3, 4). As was observed in *E. coli*, TG-resistant mutants of *A. vinelandii* still transport glucose but at a much reduced rate. The TG-resistant mutants isolated in this study exhibited an increased K_m and a decreased V_{max} for glucose uptake. This change in kinetic properties for glucose transport contributed to an increased generation time for mutants growing on glucose.

We have shown that *A. vinelandii* exhibits diauxie when grown in media containing both acetate and glucose (4). The biphasic growth of *A. vinelandii* exhibited during diauxie results from the preferential use of acetate, which in turn inhibits expression of the glucose uptake system (4). Acetate in *A. vinelandii* is transported either as the undissociated acid or as an anion in exchange for OH^- (16). Krebs cycle intermediates have also been shown to be transported in *A. vinelandii* and to inhibit glucose utilization during diauxie (4, 14). Barnes (3) has shown that acetate can reduce glucose transport as much as 93%. Glucose utilization of the wild-type *A. vinelandii* normally is transiently repressed during the diauxic lag. However, one noticeable characteristic of the mutants isolated in this study was the permanent repression of the glucose system during acetate-glucose diauxie. Revertants of TG-resistant strains were spontaneously selected. All revertants demonstrated normal growth on glucose and transient repression of the glucose system during acetate-glucose diauxie. It therefore appears these two dysfunctions of the TG-resistant strains are due to a single mutational event.

Our data suggest that acetate or an intermediate of acetate metabolism acts to repress the glucose system in *A. vinelandii*. As long as acetate is in the medium it excludes glucose uptake (i.e., inducer exclusion), preventing the

induction of the glucose system (4, 12). Increasing the exogenous concentrations of glucose during diauxic physiologically restored normal diauxic growth in the glucose transport mutant AM2. We envision that high glucose concentrations can override the inducer exclusion and the repression caused by acetate. This conclusion is also supported by the observation that increasing levels of glucose during diauxie caused the wild type not to exhibit the characteristic diauxic lag. Data from our laboratory have allowed us to propose a working hypothesis for acetate regulation of the glucose system during diauxie. We envision acetate acting as a corepressor which binds to an aporepressor and this complex acting to repress the glucose system. The aporepressor could bind glucose with a very low affinity in the presence of acetate. Depletion of acetate from diauxic media or increased glucose concentrations might result in a more efficient binding of glucose to the aporepressor and result in the induction of the glucose system. Various aspects of this model are presently under investigation in our laboratory.

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